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**ATHEROSCLEROSIS SUSCEPTIBILITY GENE LOCUS 1 (ATHSQ1) AND**  
**ATHEROSCLEROSIS SUSCEPTIBILITY GENE LOCUS 2 (ATHSQ2)**

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The invention disclosed herein was made with Government support under grant numbers HL-09930, HL-54591, and HL-22682 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly,  
10 the U.S. Government has certain rights in this invention.

**Background of the Invention**

Throughout this application, various publications are  
15 referenced by author and year. Citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to  
20 describe more fully the art to which this invention pertains.

^ ^ ^  
The genetics of atherosclerosis has been the focus of intense investigation. A subset of cases is caused by  
25 uncommon Mendelian mutations that predispose individuals to atherosclerosis (Breslow 2000; Keating and Sanguinetti 1996; Lifton 1996). The mutated genes include low-density lipoprotein receptor (LDLR) (Hobbs et al. 1992), cystathionine beta-synthase (CBS) (Kraus 1999), and, in  
30 some cases, ATP-binding cassette-A1 (Bodzioch et al. 1999; Brooks-Wilson et al. 1999; Rust et al. 1999) among others. Identification of these genes has shed light on biochemical pathways involved in atherogenesis and provided the basis for current therapeutic interventions.  
35 However, the common forms of atherosclerosis are multifactorial in origin. Attempts to map the common

susceptibility loci have been hampered by genetic heterogeneity, polygenic inheritance, incomplete pedigrees, and environmental influences. The fact that few of the genome-wide linkage studies have reported loci with large effects points to the existence of multiple loci each having small to moderate effects (Aouizerat et al. 1999; Hixson and Blangero 2000; Rice et al. 2000; Shearman 2000). The modest nature of susceptibility gene effects will likely require extremely large sample sizes or very densely-spaced genetic markers for successful linkage mapping (Risch and Merikangas 1996).

Mouse models offer significant advantages for genetic dissection of complex diseases. The ability to perform selective breeding, produce many offspring, determine inheritance of alleles without ambiguity, and control the environment is a critical factor. Early studies of murine atherosclerosis indicated that there was a clear genetic component. Inbred strains of mice exhibited a spectrum of aortic fatty streak lesion areas following the feeding of atherogenic diets high in cholesterol, fat, and cholic acid (Paigen et al. 1985; Qiao et al. 1994; Roberts and Thompson 1977). A number of susceptibility loci (*Ath1-8*) were reported based on phenotypic analyses of recombinant inbred strains derived from "resistant" and "susceptible" parents (Paigen 1995; Paigen et al. 1987, 1989; Stewart-Phillips et al. 1989). Although these studies were instrumental in pointing out strain-specific variations, none of the loci have been confirmed by more rigorous analyses of large genetic crosses.

A shortcoming of the diet-fed, inbred mouse model (in terms of carrying out quantitative genetic studies) is that aortic lesion development is minimal even in

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**Summary of the Invention**

This invention provides an isolated nucleic acid encoding a mammalian LOX-1 receptor protein, wherein the receptor protein comprises consecutive amino acids having the following sequence: -S, X, X, E, L, K, X, X, I, X, T, X, X, X, K, L, X, E, K, S, K, E, Q, X, E, L, X, X, X, X, X, N, L, Q, E, X, L, X, R, X, A, N, X, S- (SEQ ID NO: 39), wherein X is any amino acid.

The invention provides an isolated nucleic acid encoding a mammalian membrane-bound LOX-1 receptor protein, wherein the nucleic acid encodes a protein selected from the group consisting of:

- (a) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 1 in SEQ ID NO: 20,
- (b) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 3 in SEQ ID NO: 24, and
- (c) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 4 in SEQ ID NO: 26.

The invention provides an isolated nucleic acid encoding a mammalian soluble LOX-1 receptor protein, wherein the nucleic acid encodes a protein selected from the group consisting of:

- (a) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 7 in SEQ ID NO: 14,
- (b) a LOX-1 receptor protein comprising consecutive

amino acids having a sequence identical to that set forth for Isoform 8 in SEQ ID NO: 16, and

- 5 (c) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 9 in SEQ ID NO: 18.

10 The invention provides an isolated nucleic acid encoding a mammalian LOX-1 receptor protein, wherein the nucleic acid comprises:

- 15 (a) a nucleic acid sequence given in any one of SEQ ID Nos: 13, 15, 17, 19, 21, 23, 25, 27, or 28; or

- (b) a nucleic acid sequence degenerate to a sequence of (a) as a result of the genetic code.

20 The invention provides a method involving competitive binding for identifying a chemical compound which specifically binds to a mammalian LOX-1 receptor, which comprises contacting cells expressing on their cell surface the mammalian LOX-1 receptor with both the  
25 chemical compound and a second chemical compound known to bind to the receptor, and separately with only the second chemical compound, under conditions suitable for binding of both compounds, and measuring specific binding of the second chemical compound to the mammalian LOX-1 receptor,  
30 a decrease in the binding of the second chemical compound to the mammalian LOX-1 receptor in the presence of the chemical compound indicating that the chemical compound binds to the-mammalian LOX-1 receptor.

35 The invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian LOX-1 receptor to identify a compound which specifically

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binds to the mammalian LOX-1 receptor, which comprises:

- 5 (a) contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the plurality of compounds not known to bind specifically to the mammalian LOX-1 receptor, under conditions permitting binding of compounds known to bind to the mammalian LOX-1 receptor;
- 10 (b) determining whether the binding of a compound known to bind to the mammalian LOX-1 receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so
- 15 (c) separately determining the binding to the mammalian LOX-1 receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian LOX-1 receptor.
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25 The invention provides a method of identifying a compound which activates a mammalian LOX-1 receptor which comprises contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the compound under conditions permitting activation of the LOX-1 receptor, and detecting activation of the LOX-1 receptor, thereby identifying the compound as a compound which

30 activates a mammalian LOX-1 receptor.

35 The invention provides a method of identifying a compound which inhibits the activity of a mammalian LOX-1 receptor which comprises contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the compound under conditions permitting inhibition of the activity of

the LOX-1 receptor, and detecting inhibition of the activity of the LOX-1 receptor, thereby identifying the compound as a compound which inhibits the activity of a mammalian LOX-1 receptor.

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The invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian LOX-1 receptor to identify a compound which activates the mammalian LOX-1 receptor which comprises:

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(a) contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the plurality of compounds not known to activate the mammalian LOX-1 receptor, under conditions permitting activation of the mammalian LOX-1 receptor;

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(b) determining whether the activity of the mammalian LOX-1 receptor is increased in the presence of the compounds; and if so

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(c) separately determining whether the activation of the mammalian LOX-1 receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound which activates the mammalian LOX-1 receptor.

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The invention provides a method of screening a plurality of chemical compounds not known to inhibit the activity of a mammalian LOX-1 receptor to identify a compound which inhibits the activity of the mammalian LOX-1 receptor, which comprises:

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(a) contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the plurality of compounds in the presence of a

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5 (b) determining whether the activity of the  
mammalian LOX-1 receptor is reduced in the  
presence of the plurality of compounds,  
relative to the activity of the mammalian LOX-1  
receptor in the absence of the plurality of  
10 compounds; and if so

20 The invention provides a method of treating or preventing atherosclerosis in a subject which comprises administering to the subject an amount of a compound effective to decrease the activity of a mammalian LOX-1 receptor and treat atherosclerosis in the subject.

The invention provides a method of treating inflammation in a subject which comprises administering to the subject an amount of a soluble mammalian LOX-1 receptor effective to treat inflammation in the subject.

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### Brief Description of the Figures

**Figure 2.** LOD score plots for Chr 4 and Chr 6 lesion susceptibility QTLs. The y-axis indicates LOD scores; the x-axis indicates position along the chromosome (distance from the centromere in centiMorgans, cM). Microsatellite markers typed in Mbc-Ldlr0 mice are indicated below the x-axis. LOD scores were calculated and plotted at 2-cM intervals using Map Manager QT software. The significance threshold of  $p = 0.05$  for a backcross is indicated by a solid line at  $\text{LOD} = 3.3$ .

Figure 3A-3C. Sequence alignment of mouse LOX-1 coding regions. LOX-1C primers were used to clone LOX-1 coding regions from macrophage cDNAs by polymerase chain reaction. Alignment in DIALIGN format.

Aligned sequences:

B-Isoform 1 (B6-Isoform 1), rat lox-like

(SEQ ID NO: 11);

M-Isoform 1 (MOLF-Isoform 1), rat lox-like

(SEQ ID NO: 12);

Isoform 7, soluble (SEQ ID NO: 13);

Isoform 8, soluble (SEQ ID NO: 15);

Isoform 9, soluble (SEQ ID NO: 17).

TM = transmembrane domain. 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> repeat = copies of a unique repetitive region.

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Figure 12. Nucleotide and amino acid sequences for LOX-1 Isoform 9 (SEQ ID NO: 17 and 18, respectively).

Figure 13A-13E. Alignment of amino acid sequences of LOX-1 repeat motifs.

A. Alignment of 46 amino acid repeat motifs (R1, R2, and R3) for Isoforms 1, 3, 4, 7, and 8. The sequence for Isoform 3 is incomplete. Isoforms 2, 5, 6, and 9 do not

contain repeats. The dashed lines beneath the sequence alignment indicate positions where there is 100% identity among the sequences. Isoform 1 (R1), SEQ ID NO: 29; Isoform 1 (R2), SEQ ID NO: 30; Isoform 1 (R3), SEQ ID NO: 31; Isoform 3 (R1), SEQ ID NO: 32; Isoform 3 (R3), SEQ ID NO: 33; Isoform 4 (R1), SEQ ID NO: 34; Isoform 7 (R2), SEQ ID NO: 35; Isoform 7 (R3), SEQ ID NO: 36; Isoform 8 (R3), SEQ ID NO: 37.

10 B.-D. The sequences from A are aligned for repeat 1 (R1) in B, repeat 2 (R2) in C, and repeat 3 (R3) in D.

15 E. The repeat motifs encoded by macrophage-derived isoforms of mouse LOX-1 from A are aligned with a homologous region encoded by endothelial-derived human LOX-1. The human region (SEQ ID NO: 38) does not repeat. Human sequence from Sawamura et al. (1997).

20 Figure 14. Probability of regions of the LOX-1 sequence forming coiled coil structures. The repeat units of LOX-1 are predicted to form highly conserved coiled coil structures. The probability plot for Isoform 1 is shown. Figure generated using COILS software (described in Lupas et al. 1991, 1996).

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Detailed Description of the Invention

Throughout this application, the following standard abbreviations are used to indicate specific amino acids:

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	3-character abbreviation	Amino Acid	1-character abbreviation
	Ala	Alanine	A
10	Arg	Arginine	R
	Asn	Asparagine	N
	Asp	Aspartic Acid	D
	Cys	Cysteine	C
	Gln	Glutamine	Q
15	Glu	Glutamic Acid	E
	Gly	Glycine	G
	His	Histidine	H
	Ile	Isoleucine	I
	Leu	Leucine	L
20	Lys	Lysine	K
	Met	Methionine	M
	Phe	Phenylalanine	F
	Pro	Proline	P
	Ser	Serine	S
25	Thr	Threonine	T
	Trp	Tryptophane	W
	Tyr	Tyrosine	Y
	Val	Valine	V
	Asx	Asparagine/	B
30		Aspartic Acid	
	Glx	Glutamine/ Glutamic Acid	Z
	***	(End)	*
35	Xxx	Any amino acid or as specified.	X

The following standard abbreviations are used to indicate specific nucleotide bases:

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A = adenine;  
C = cytosine;  
G = guanine;  
T = thymine.

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The following definitions are presented as an aid in understanding this invention:

Chr, chromosome;  
5 cM, centiMorgans;  
HDL, high density lipoprotein;  
LDL, low density lipoprotein;  
Ldlr, low density lipoprotein receptor;  
LOD, logarithm of odds;  
10 LOX-1, oxidized low density lipoprotein receptor  
Olrl, oxidized low density lipoprotein receptor  
MGD, Mouse Genome Database;  
QTL, quantitative trait locus.

15 "inhibiting LOX-1 activity", examples include, without  
limitation, interfering with or blocking ligand binding  
to and activation of the receptor;

20 "treating" a subject, examples include, without  
limitation, reversing, slowing, stabilizing or otherwise  
ameliorating a disease or disorder with which the subject  
is afflicted;

25 "inhibit onset" of a disorder, examples include, without  
limitation, lessening the likelihood of onset, delaying  
the onset, or preventing the onset.

30 Having due regard to the preceding definitions, this  
invention provides an isolated nucleic acid encoding a  
protein comprising an amino acid sequence selected from  
the group consisting of SEQ ID NO:14, SEQ ID NO:16, SEQ  
ID NO: 18, SEQ ID NO:22, SEQ ID NO:24, and SEQ ID NO:26.  
In different embodiments, the nucleic acid has a sequence  
selected from the group consisting of SEQ ID NO:13, SEQ  
35 ID NO:15, SEQ ID NO:17, SEQ ID NO:21, SEQ ID NO:23, SEQ

ID NO:25, SEQ ID NO:27, and SEQ ID NO:28.

The invention provides an isolated nucleic acid encoding a mammalian LOX-1 receptor protein, wherein the receptor protein comprises consecutive amino acids having the following sequence: -S, X, X, E, L, K, X, X, I, X, T, X, X, X, K, L, X, E, K, S, K, E, Q, X, E, L, X, X, X, X, X, N, L, Q, E, X, L, X, R, X, A, N, X, S- (SEQ ID NO: 39), wherein X is any amino acid.

In one embodiment, the receptor protein comprises consecutive amino acids having the following sequence: -S, K or Q or E, K or R or N, E, L, K, G or E, K or M, I, D or E, T, L or I, T or A, Q or R or L, K, L, N or D, E, K, S, K, E, Q, E or M, E, L, L or H, Q or H, K or M or Q, N or I, Q or L, N, L, Q, E, A or T, L, Q or K, R, A or V, A, N, S or F or C, S- (SEQ ID NO: 40).

The invention provides an isolated nucleic acid encoding a mammalian membrane-bound LOX-1 receptor protein, wherein the nucleic acid encodes a protein selected from the group consisting of:

- (a) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 1 in SEQ ID NO: 20,
- (b) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 3 in SEQ ID NO: 24, and
- (c) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 4 in SEQ ID NO: 26.

The invention provides an isolated nucleic acid encoding a mammalian soluble LOX-1 receptor protein, wherein the



nucleic acid encodes a protein selected from the group consisting of:

(a) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 7 in SEQ ID NO: 14,

(b) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 8 in SEQ ID NO: 16, and

(c) a LOX-1 receptor protein comprising consecutive amino acids having a sequence identical to that set forth for Isoform 9 in SEQ ID NO: 18.

The invention provides an isolated nucleic acid encoding a mammalian LOX-1 receptor protein, wherein the nucleic acid comprises:

(a) a nucleic acid sequence given in any one of SEQ ID Nos: 13, 15, 17, 19, 21, 23, 25, 27, or 28; or

(b) a nucleic acid sequence degenerate to a sequence of (a) as a result of the genetic code.

In different embodiments of any of the isolated nucleic acids described herein, the nucleic acid is DNA or RNA. In different embodiments, the DNA is cDNA, genomic DNA, or synthetic DNA.

In one embodiment of any of the isolated nucleic acids described herein, the nucleic acid molecule encodes a mouse LOX-1 receptor or a human LOX-1 receptor.

This invention provides a nucleic acid probe of at least about 15 nucleotides in length which specifically hybridizes with a nucleic acid encoding a mammalian LOX-1

receptor or with a nucleic acid having the complementary sequence thereof. In different embodiments of the probe, the mammalian LOX-1 receptor has an amino acid sequence selected from the group consisting of SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO: 18, SEQ ID NO:22, SEQ ID NO:24, and SEQ ID NO:26. In different embodiments, the probe specifically hybridizes with a nucleic acid encoding the amino acid sequence shown in SEQ ID NO:39. In different embodiments, the probe is labeled with a detectable marker.

This invention provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with and has a sequence complementary to a unique sequence present within (a) any one of the nucleic acids described herein or (b) the reverse complement thereof. In different embodiments, the nucleic acid probe is DNA, cDNA, genomic DNA, synthetic DNA or RNA.

As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

This invention provides an isolated protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO: 18, SEQ ID NO:22, SEQ ID NO:24, and SEQ ID NO:26.

This invention provides a purified mammalian membrane-bound LOX-1 receptor protein, wherein the protein comprises consecutive amino acids having a sequence identical to the sequence set forth for Isoform 1 in SEQ ID NO: 20, or for Isoform 3 in SEQ ID NO: 24, or for Isoform 4 in SEQ ID NO: 26.

This invention provides a purified mammalian soluble LOX-1 receptor protein, wherein the protein comprises consecutive amino acids having a sequence identical to the sequence set forth for Isoform 7 in SEQ ID NO: 14, or for Isoform 8 in SEQ ID NO: 16, or for Isoform 9 in SEQ ID NO: 18.

The invention provides a purified mammalian LOX-1 receptor protein encoded by any of the isolated nucleic acids described herein.

The invention provides a method of preparing a purified mammalian LOX-1 receptor protein which comprises:

- (a) inserting any of the isolated nucleic acids encoding the protein described herein into a suitable expression vector;
- (b) introducing the resulting vector into a suitable host cell;
- (c) placing the resulting host cell in suitable conditions permitting the production of the protein;
- (d) recovering the protein so produced; and optionally
- (e) isolating and/or purifying the protein so recovered.

This invention provides a vector comprising any of the nucleic acids described herein. In different embodiments, the vector is adapted for expression of the nucleic acid in a cell and comprises regulatory elements necessary for expression of the nucleic acid in the cell operatively linked to the nucleic acid so as to permit expression thereof. In different embodiments, the cell is a bacterial, Archaeal, amphibian, yeast, fungal, insect, plant, or mammalian cell. In different embodiments, the vector is a plasmid, a baculovirus, retrovirus, or a bacteriophage.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art.

The invention provides a method of transforming a cell which comprises transfecting a host cell with any of the vectors described herein.

This invention provides a cell comprising any of the vectors described herein. This invention provides a membrane preparation isolated from any of the herein described cells. This invention also provides a soluble extract isolated from any of the herein described cells. In different embodiments, the cell is a bacterial, Archaeal, amphibian, yeast, fungal, insect, plant, or mammalian cell. In different embodiments, the amphibian cell is a *Xenopus* oocyte cell or a *Xenopus* melanophore cell. In different embodiments, the mammalian cell is a HEK293 cell, a Chinese hamster ovary (CHO) cell, a COS-7 cell, a LM(tk-) cell, a mouse embryonic fibroblast NIH-3T3 cell, a mouse Y1 cell, a 293 human embryonic kidney cell, or a HeLa cell. In different embodiments, the insect cell is an Sf9 cell, an Sf21 cell or a *Trichoplusia ni* 5B-4 cell.

In one embodiment, prior to being transfected with the

vector the host cell does not express a mammalian LOX-1 receptor protein. In one embodiment, prior to being transfected with the vector the host cell does express a mammalian LOX-1 receptor protein. In one embodiment, but  
5 for the vector present therein, the cell would not express a mammalian LOX-1 receptor.

Methods of transforming and transfecting cells with nucleic acid to obtain cells in which the encoded protein  
10 is expressed are well known in the art (Sambrook et al. 1989). Such transformed cells may also be used to test compounds and screen compound libraries to obtain compounds which bind to the expressed protein and therefore are likely to do so *in vivo*.

15 DNA encoding proteins to be studied, including foreign proteins, can be expressed by several methods. Heterologous DNA can be stably incorporated into host cells, causing the cell to perpetually express a foreign protein. DNA to be expressed can be introduced on  
20 plasmid or bacteriophage vectors by transformation or transfection (including treatment of cells with  $MgCl_2$  or  $CaCl_2$ , electroporation, or natural transformation), conjugation, or transduction, often, but not necessarily,  
25 following selection for linked antibiotic resistance genes. The ensuing drug resistance can be exploited to select and maintain cells that have taken up the heterologous DNA. An assortment of resistance genes are available including but not restricted to Neomycin, Kanamycin, and Hygromycin. Genes for proteins to be  
30 studied may be expressed constitutively or their expression may be induced from regulated promoters. DNA to be expressed may be located on extrachromosomal elements, such as plasmids, on integrated prophages, or  
35 inserted into chromosomes by homologous recombination or

transposition. DNA encoding proteins to be studied can also be transiently expressed in a variety of mammalian, insect, amphibian, yeast, fungal, plant and other cells by several methods, including but not restricted to transformation, transfection, calcium phosphate-mediated, DEAE-dextran mediated, liposomal-mediated, viral-mediated, electroporation-mediated and microinjection delivery. Each of these methods may require optimization of assorted experimental parameters depending on the DNA, cell line, and the type of assay to be subsequently employed.

The invention provides an antisense oligonucleotide which specifically hybridizes to any of the RNA described herein, so as to prevent translation of the RNA. The invention provides an antisense oligonucleotide which specifically hybridizes to any of the DNA described herein. In one embodiment, the antisense oligonucleotide comprises chemically modified nucleotides or nucleotide analogues.

<sup>A</sup>This invention provides an antibody capable of binding to any of the proteins described herein. In one embodiment, the antibody is a monoclonal antibody. In one embodiment, the antibody is a polyclonal antibody.

The invention provides a transgenic, nonhuman mammal expressing DNA encoding any of the mammalian LOX-1 receptors described herein. The invention provides a transgenic, nonhuman mammal comprising a homologous recombination knockout of a native LOX-1 receptor.

The invention provides a method of identifying a compound which specifically binds to a mammalian LOX-1 receptor protein which comprises contacting any of the purified LOX-1 receptor proteins described herein with the

compound under conditions permitting binding of the compound to the purified LOX-1 receptor protein, and detecting the presence of any such compound specifically bound to the receptor protein, thereby identifying the compound as a compound which specifically binds to a mammalian LOX-1 receptor protein. In one embodiment, the purified LOX-1 receptor protein is embedded in a lipid bilayer.

The invention provides a method of determining whether an agent inhibits the activity of a membrane-bound mammalian LOX-1 receptor, which comprises (a) contacting the agent with the receptor under conditions which would permit the inhibition of such activity by an activity-inhibiting agent, and (b) detecting whether the agent has inhibited the activity of the LOX-1 receptor. In one embodiment, the LOX-1 receptor is a mouse receptor. In one embodiment, the LOX-1 receptor is a human receptor.

The invention provides an agent determined by any of the methods described herein to inhibit the activity of a membrane-bound mammalian LOX-1 receptor. The invention provides a composition which comprises the agent and a pharmaceutically acceptable carrier.

The invention provides a method of preparing a composition which comprises identifying an agent by any of the methods described herein, recovering the agent free of LOX-1 receptor, and admixing the agent with a pharmaceutically acceptable carrier.

The invention provides a method of identifying a compound which specifically binds to a mammalian LOX-1 receptor which comprises contacting cells expressing the LOX-1 receptor, or a membrane fraction or a soluble fraction from said cells, with the compound under conditions permitting binding of the compound to the LOX-1 receptor,

and detecting the presence of any such compound specifically bound to the receptor, thereby identifying the compound as a compound which specifically binds to a mammalian LOX-1 receptor.

In one embodiment of any of the methods described herein, the cells do not normally express the mammalian LOX-1 receptor and the mammalian LOX-1 receptor is encoded by any of the isolated nucleic acids described herein.

The invention provides a method involving competitive binding for identifying a chemical compound which specifically binds to a mammalian LOX-1 receptor which comprises contacting cells expressing on their cell surface the mammalian LOX-1 receptor with both the chemical compound and a second chemical compound known to bind to the receptor, and separately with only the second chemical compound, under conditions suitable for binding of both compounds, and measuring specific binding of the second chemical compound to the mammalian LOX-1 receptor, a decrease in the binding of the second chemical compound to the mammalian LOX-1 receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian LOX-1 receptor. In one embodiment, the second chemical compound is labeled oxidized-LDL. In one embodiment, the binding of the second chemical compound to the LOX-1 receptor is measured by quantifying the amount of labeled oxidized-LDL inside the cells. In different embodiments, oxidized-LDL is labeled with a fluorescent label, a radioactive label, or a colorimetric label. In one embodiment, oxidized-LDL is labeled with  $^3\text{H}$ . In one embodiment, the cells do not normally express the mammalian LOX-1 receptor and the mammalian LOX-1 receptor is encoded by any of the isolated nucleic acids described herein.

[illegible]



The invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian LOX-1 receptor to identify a compound which specifically binds to the mammalian LOX-1 receptor, which comprises:

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(a) contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the plurality of compounds not known to bind specifically to the mammalian LOX-1 receptor, under conditions permitting binding of compounds known to bind to the mammalian LOX-1 receptor;

10

(b) determining whether the binding of a compound known to bind to the mammalian LOX-1 receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so

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(c) separately determining the binding to the mammalian LOX-1 receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian LOX-1 receptor.

25

In one embodiment of any of the methods described herein, the compound known to bind to the mammalian LOX-1 receptor is labeled oxidized-LDL. In one embodiment, the binding of labeled oxidized-LDL to the LOX-1 receptor is measured by quantifying the amount of labeled oxidized-LDL inside the cells. In different embodiments, oxidized-LDL is labeled with a fluorescent label, a radioactive label, or a colorimetric label. In one embodiment, oxidized-LDL is labeled with <sup>3</sup>H. In one embodiment, the cells do not normally express the mammalian LOX-1 receptor and the mammalian LOX-1 receptor

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is encoded by any of the isolated nucleic acids described herein.

5 The invention provides a method involving competitive binding for identifying a chemical compound which specifically binds to a mammalian soluble LOX-1 receptor which comprises contacting the mammalian soluble LOX-1 receptor with both the chemical compound and a second chemical compound known to bind to the receptor, and  
10 separately with only the second chemical compound, under conditions suitable for binding of both compounds, and measuring specific binding of the second chemical compound to the mammalian soluble LOX-1 receptor, a decrease in the binding of the second chemical compound  
15 to the mammalian soluble LOX-1 receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian soluble LOX-1 receptor. In one embodiment, the mammalian soluble LOX-1 receptor is immobilized on a solid surface. In one embodiment,  
20 the second chemical compound is labeled oxidized-LDL. In different embodiments, oxidized-LDL is labeled with a fluorescent label, a radioactive label, or a colorimetric label. In one embodiment, oxidized-LDL is labeled with <sup>3</sup>H. In one embodiment, the mammalian soluble LOX-1  
25 receptor is encoded by any of the isolated nucleic acids described herein.

30 The invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian soluble LOX-1 receptor to identify a compound which specifically binds to the mammalian soluble LOX-1 receptor, which comprises:

- 35 (a) contacting the mammalian soluble LOX-1 receptor with the plurality of compounds not known to bind specifically to the mammalian soluble LOX-1 receptor, under conditions permitting binding

of compounds known to bind to the mammalian soluble LOX-1 receptor;

5 (b) determining whether the binding of a compound known to bind to the mammalian soluble LOX-1 receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if  
10 so

15 (c) separately determining the binding to the mammalian soluble LOX-1 receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian soluble LOX-1 receptor.

20 In one embodiment of any of the methods described herein, the compound known to bind to the mammalian soluble LOX-1 receptor is labeled oxidized-LDL. In different embodiments, oxidized-LDL is labeled with a fluorescent label, a radioactive<sup>^</sup> label, or a colorimetric label<sup>^</sup>. In one embodiment, oxidized-LDL is labeled with <sup>3</sup>H. In one  
25 embodiment, the mammalian soluble LOX-1 receptor is encoded by any of the isolated nucleic acids described herein. In one embodiment, the mammalian soluble LOX-1 receptor is immobilized on a solid surface.

30 The invention provides a method of identifying a compound which activates a mammalian LOX-1 receptor which comprises contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the compound under conditions permitting activation of the LOX-1  
35 receptor, and detecting activation of the LOX-1 receptor, thereby identifying the compound as a compound which activates a mammalian LOX-1 receptor. In one embodiment,

the cells do not normally express the mammalian LOX-1 receptor and the mammalian LOX-1 receptor is encoded by any of the isolated nucleic acids described herein.

5 The invention provides a method of identifying a compound which inhibits the activity of a mammalian LOX-1 receptor which comprises contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the compound under conditions permitting inhibition of the activity of  
10 the LOX-1 receptor, and detecting inhibition of the activity of the LOX-1 receptor, thereby identifying the compound as a compound which inhibits the activity of a mammalian LOX-1 receptor. In one embodiment, the cells do not normally express the mammalian LOX-1 receptor and  
15 the mammalian LOX-1 receptor is encoded by any of the isolated nucleic acids described herein.

The invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian  
20 LOX-1 receptor to identify a compound which activates the mammalian LOX-1 receptor which comprises:

- 25 (a) contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the plurality of compounds not known to activate the mammalian LOX-1 receptor, under conditions permitting activation of the mammalian LOX-1 receptor;
- 30 (b) determining whether the activity of the mammalian LOX-1 receptor is increased in the presence of the compounds; and if so
- 35 (c) separately determining whether the activation of the mammalian LOX-1 receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the

compound which activates the mammalian LOX-1 receptor.

The invention provides a method of screening a plurality of chemical compounds not known to inhibit the activity of a mammalian LOX-1 receptor to identify a compound which inhibits the activity of the mammalian LOX-1 receptor, which comprises:

(a) contacting cells expressing on their cell surface the mammalian LOX-1 receptor with the plurality of compounds in the presence of a known compound which activates the mammalian LOX-1 receptor, under conditions permitting activation of the mammalian LOX-1 receptor;

(b) determining whether the activity of the mammalian LOX-1 receptor is reduced in the presence of the plurality of compounds, relative to the activity of the mammalian LOX-1 receptor in the absence of the plurality of compounds; and if so

(c) separately determining the inhibition of activity of the mammalian LOX-1 receptor for each compound included in the plurality of compounds, so as to thereby identify the compound which inhibits the activity of the mammalian LOX-1 receptor.

In one embodiment of any of the methods described herein, the known compound which activates the mammalian LOX-1 receptor is oxidized-LDL.

In one embodiment of any of the methods described herein, the cells do not normally express the mammalian LOX-1 receptor and the mammalian LOX-1 receptor is encoded by

any of the isolated nucleic acids described herein. In one embodiment of any of the methods described herein, prior to being transfected with a vector comprising any of the nucleic acids described herein, the cells do not express a mammalian LOX-1 receptor protein. In one embodiment of any of the methods described herein, the cells do not express the mammalian LOX-1 receptor prior to being transfected with nucleic acid encoding the mammalian LOX-1 receptor, wherein the nucleic acid comprises any of the isolated nucleic acids described herein. In one embodiment of any of the methods described herein, the cells do not express the mammalian LOX-1 receptor prior to being transfected with nucleic acid encoding the mammalian LOX-1 receptor, wherein the mammalian LOX-1 receptor comprises consecutive amino acids having the following sequence: -S, X, X, E, L, K, X, X, I, X, T, X, X, X, K, L, X, E, K, S, K, E, Q, X, E, L, X, X, X, X, X, N, L, Q, E, X, L, X, R, X, A, N, X, S- (SEQ ID NO: 39), wherein X is any amino acid.

The activity of the LOX-1 receptor can be detected in different ways. In one embodiment, activation of the LOX-1 receptor is detected by measuring increased intracellular reactive oxygen species production (Cominacini et al. 2000). In one embodiment, activation of the LOX-1 receptor is detected by measuring increased activation of the transcription factor Nuclear Factor-kappaB (NF-KB) (Cominacini et al. 2000). In one embodiment, activation of the LOX-1 receptor is detected by measuring increased monocyte chemoattractant protein-1 (MCP-1) gene expression (Li and Mehta 2000). Conversely, inhibition of the activity of the LOX-1 receptor is detected by measuring a decrease in any one of the parameters recited above.

In one embodiment of any of the methods described herein,

The invention provides a method of reducing the amount of a mammalian LOX-1 receptor on the surface of a cell, which comprises delivering to the cell an agent that  
35 reduces the expression of mammalian LOX-1 receptor therein. In different embodiments, the agent is a catalytic nucleic acid or an antisense nucleic acid. In

one embodiment, the agent is a ribozyme.

The invention provides a method of treating a mammalian subject afflicted with a disorder selected from the group consisting of atherosclerosis, heart failure and stroke, comprising administering to the subject a therapeutically effective amount of an agent that inhibits the activity of LOX-1 receptors in the subject.

The invention provides a method of treating a mammalian subject afflicted with a disorder selected from the group consisting of atherosclerosis, heart failure and stroke, comprising administering to the subject a therapeutically effective amount of an agent that inhibits the expression of LOX-1 receptors in the subject's cells. In different embodiments, the agent is a catalytic nucleic acid or an antisense nucleic acid. In one embodiment, the agent is a ribozyme.



subject's cells. In different embodiments, the agent is a catalytic nucleic acid or an antisense nucleic acid. In one embodiment, the agent is a ribozyme.

10           The invention provides a method of inhibiting the onset  
in a mammalian subject of a disorder selected from the  
group consisting of atherosclerosis, heart failure and  
stroke, comprising administering to the subject a  
15       prophylactically effective amount of a soluble LOX-1  
receptor.

In one embodiment of any of the methods described herein,  
the subject is a mouse. In one embodiment, the subject  
is a human.

The invention provides a method of preventing atherosclerosis in a subject which comprises administering to the subject an amount of a soluble mammalian LOX-1 receptor effective to prevent

atherosclerosis in the subject. In one embodiment, the subject is known to be susceptible to atherosclerosis.

5 In one embodiment of any of the methods described herein, the soluble LOX-1 receptor binds LOX-1 receptor ligand and reduces availability of LOX-1 receptor ligand to bind to a membrane-bound LOX-1 receptor.

10 The invention provides a method of treating atherosclerosis in a subject which comprises administering to the subject an amount of a compound effective to decrease the activity of a mammalian LOX-1 receptor and treat atherosclerosis in the subject. In one embodiment, the LOX-1 receptor is a membrane-bound  
15 Lox-1 receptor.

The invention provides a method of preventing atherosclerosis in a subject which comprises administering to the subject an amount of a compound  
20 effective to decrease the activity of a mammalian LOX-1 receptor and prevent atherosclerosis in the subject. In one embodiment, the LOX-1 receptor is a membrane-bound Lox-1 receptor. In one embodiment, the subject is known  
25 to be susceptible to atherosclerosis.

This invention provides a method of determining the susceptibility of a subject to atherosclerosis, which comprises detecting soluble LOX-1 receptor in the subject's plasma, wherein the presence of soluble LOX-1  
30 receptor indicates an decreased susceptibility to atherosclerosis. This invention provides a method of determining the susceptibility of a subject to atherosclerosis, which comprises detecting soluble LOX-1 receptor in the subject's plasma, wherein an absence of  
35 soluble LOX-1 receptor indicates an increased

susceptibility to atherosclerosis.

The invention provides a method of treating inflammation in a subject which comprises administering to the subject an amount of a soluble mammalian LOX-1 receptor effective to treat inflammation in the subject. In one embodiment, the soluble LOX-1 receptor binds LOX-1 receptor ligand and reduces availability of LOX-1 receptor ligand to bind to a membrane-bound LOX-1 receptor.

The invention provides a method of treating inflammation in a subject which comprises administering to the subject an amount of a compound effective to decrease the activity of a mammalian LOX-1 receptor and treat inflammation in the subject. In one embodiment, the LOX-1 receptor is a membrane-bound Lox-1 receptor.

The invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian LOX-1 receptor, which comprises administering to the subject an amount of a compound effective to decrease the activity of the LOX-1 receptor, thereby treating the abnormality. In one embodiment the LOX-1 receptor is a membrane-bound LOX-1 receptor. In one embodiment the abnormality is atherosclerosis. In one embodiment the abnormality is inflammation. In one embodiment the abnormality is heart disease. In one embodiment the abnormality is stroke.

The invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing LOX-1 signal transduction, which comprises administering to the subject an amount of a soluble mammalian LOX-1 receptor effective to bind LOX-1 receptor ligand and reduce availability of LOX-1 receptor

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ligand to bind to a membrane-bound LOX-1 receptor, thereby decreasing LOX-1 signal transduction and treating the abnormality. In one embodiment the abnormality is atherosclerosis. In one embodiment the abnormality is inflammation. In one embodiment the abnormality is heart disease. In one embodiment the abnormality is stroke.

In one embodiment of any of the methods described herein, the subject is a human. In one embodiment of any of the methods described herein, the mammalian LOX-1 receptor is encoded by any of the nucleic acids described herein. In one embodiment of any of the methods described herein, the compound is identified by any of the methods described herein.

The invention provides for the use of a chemical compound identified by any of the methods described herein for the preparation of a pharmaceutical composition for treating an abnormality, wherein the abnormality is alleviated by decreasing the activity of a LOX-1 receptor. In one embodiment the LOX-1 receptor is a membrane-bound LOX-1 receptor. In one embodiment the abnormality is atherosclerosis. In one embodiment the abnormality is inflammation.

This invention provides a compound identified by any one of the methods described herein. In one embodiment, the compound is not previously known to bind to a mammalian LOX-1 receptor. In one embodiment, the compound is not previously known to activate a mammalian LOX-1 receptor. In one embodiment, the compound is not previously known to inhibit the activity of a mammalian LOX-1 receptor.

The invention provides a composition which comprises a compound identified by any of the methods described

herein and a carrier. This invention provides a pharmaceutical composition comprising an amount of a chemical compound identified by any of the methods described herein and a pharmaceutically acceptable carrier. The invention provides a pharmaceutical composition comprising a compound identified by a method described herein effective to increase mammalian LOX-1 receptor activity and a pharmaceutically acceptable carrier. The invention provides a pharmaceutical composition comprising a compound identified by a method described herein effective to decrease mammalian LOX-1 receptor activity and a pharmaceutically acceptable carrier.

The invention provides a method of preparing a composition which comprises identifying a compound by any of the methods described herein and admixing a carrier. Examples of carriers include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions.

The invention provides a method for preparing a composition which comprises admixing a carrier and a pharmaceutically effective amount of a chemical compound identified by any of the methods described herein or a novel structural and functional analog or homolog thereof.

This invention provides a method of preparing a composition which comprises identifying a compound by any of the methods described herein, recovering the compound free of any LOX-1 receptor or cellular components, and admixing the compound with a pharmaceutically acceptable carrier. This invention provides a method of preparing a composition which comprises determining whether a compound binds to a mammalian LOX-1 receptor using any of the methods described herein, recovering the compound

free of any LOX-1 receptor, and admixing the compound with a pharmaceutically acceptable carrier. This invention provides a method of preparing a composition which comprises determining whether a compound activates a mammalian LOX-1 receptor using any of the methods described herein, recovering the compound free of any LOX-1 receptor, and admixing the compound with a pharmaceutically acceptable carrier. This invention provides a method of preparing a composition which comprises determining whether a compound inhibits the activity of a mammalian LOX-1 receptor using any of the methods described herein, recovering the compound free of any LOX-1 receptor, and admixing the compound with a pharmaceutically acceptable carrier.

This invention provides the use of a chemical compound identified by any of the methods described herein for the preparation of a pharmaceutical composition for treating an abnormality, wherein the abnormality is alleviated by reducing the activity of a mammalian LOX-1 receptor. In one embodiment, the mammalian LOX-1 receptor is a membrane-bound LOX-1 receptor. In one embodiment, the mammalian LOX-1 receptor is a human LOX-1 receptor. In one embodiment, the abnormality is atherosclerosis. In one embodiment, the abnormality is inflammation.

In the subject invention, a "pharmaceutically or therapeutically effective amount" is any amount of a compound or agent which, when administered to a subject suffering from a disease against which the compound or agent is effective, causes reduction, remission, or regression of the disease. A "prophylactically effective amount" is any amount of a compound or agent which, when administered to a subject, inhibits the onset in the subject of a disease or disorder against which the compound or agent is effective. Furthermore, as used

herein, the phrase "pharmaceutically acceptable carrier" means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions.

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This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

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## Experimental Details

### Materials and Methods

5 Mice: MOLF/Ei (MOLF) and B6.129S7-*Ldlr*<sup>tm1Her</sup> (formerly  
C57BL/6J-*Ldlr*<sup>tm1Her</sup>; hereafter referred to as B6-Ldlr0) were  
purchased from The Jackson Laboratory (Bar Harbor, ME).  
MOLF females were mated with B6-Ldlr0 males to produce F1  
mice. Female F1s were backcrossed to B6-Ldlr0 males to  
10 produce N2 mice homozygous for the *Ldlr* knockout allele.  
N2 mice were weaned onto standard laboratory chow  
(PicoLab Rodent 20, #5053) at 21 days of age and switched  
to a Western-style diet at 8-12 weeks of age. The Western  
diet contained 21% wt/wt butterfat and 0.15% wt/wt  
cholesterol (Harlan Teklad Adjusted Calories TD 88137).  
15 Mice were bled after two weeks and three months of  
Western diet feeding, and sacrificed at the three-month  
time-point. The breeding colony was produced and  
maintained in a specific pathogen-free environment. All  
mice were given *ad libitum* access to food and water and  
20 maintained on a standard 12-h light-dark cycle throughout  
the study. All experimental protocols were approved by  
the Institutional Animal Care and Research Advisory  
Committee.

25 *Atherosclerotic lesion measurements:* Anesthetized mice  
were sacrificed by cervical dislocation. The hearts were  
perfused with 0.9% NaCl by cardiac intraventricular  
canalization. Then, the hearts and aortic root were  
dissected and fixed in 10% formalin. The aortic root was  
30 sectioned, stained with oil red O, and lesion areas were  
quantified as described by Plump et al. (1994).

*Plasma lipoprotein and insulin measurements:* Mice were  
bled in the middle of the light cycle following a 5-6



hour fast. Retro-orbital bleeding was performed under Forane anesthesia (Baxter, Deerfield, IL). Blood was collected directly into heparinized capillary tubes (Becton Dickson). Plasma was separated from cells by centrifugation and stored at -70 °C. Isolation of HDL cholesterol by chemical precipitation (HDL reagent, Sigma), as well as enzymatic measurements of cholesterol and triglycerides (Wako Pure Chemical Industries, Ltd.), were carried out according to the manufacturers' instructions. Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol. Insulin was measured using a commercially available ELISA kit (Crystal Chem, Inc., Chicago, IL).

*DNA extraction and LdlrKO genotyping:* DNA was extracted from tail tips by a quick alkaline lysis protocol (Truett et al. 2000). The tail tips were incubated in 50 mM NaOH for 1 hour at 95 °C, vortexed and neutralized in 1 M Tris (pH 8). Cellular debris was pelleted by centrifugation and the supernatant was used for polymerase chain reaction (PCR) amplification of *Ldlr* alleles. *Ldlr* for wild type allele primers (SEQ ID NOs: 1 and 2) and *Ldlr* for mutant allele primers (SEQ ID NOs: 3 and 4) were used for *Ldlr* genotyping.

*Ldlr* (wild type allele)

Forward, 5'-ACCCCAAGACGTGCTCCCAGGATGA-3' (SEQ ID NO: 1)

Reverse, 5'-CGCAGTGCTCCTCATCTGACTTGT-3' (SEQ ID NO: 2)

*Ldlr* (mutant allele)

Forward, 5'-AGGATCTCGTCTGACCCATGGCGA-3' (SEQ ID NO: 3)

Reverse, 5'-GAGCGGCGATACCGTAAAGCACGAGG-3'  
(SEQ ID NO: 4)

*Ldlr* typings were confirmed by measuring plasma

cholesterol levels.

*DNA pooling and genome scan:* DNA was quantified, in quadruplicate, by spectrophotometry. Equal amounts of DNA were pooled from 10-15 mice in the top or bottom 20% of the phenotypic ranges. Separate pools were made for males and females. The final concentration of DNA in the pools was 100-150 ng/ $\mu$ l, such that each individual sample was represented at a concentration of 10 ng/ $\mu$ l in a pool. Microsatellite markers (Dietrich et al. 1992; Love et al. 1990) were typed by PCR amplification using D4Mit127 and D6Mit110 primers purchased from Research Genetics (Huntsville, AL). D4Mit127 primer was used to detect linkage to *Athsq1*, and D6Mit110 primer was used to detect linkage to *Athsq2*:

PCR products were separated on 7% Long Ranger polyacrylamide (FMC BioProducts) gels and scored using a LI-COR Model 4000S automated DNA sequencer (Lincoln, NE) and Gene ImagIR v3.55 software (Scanalytics, Billerica, MA). Parental and F1 DNA samples were run alongside the pools as controls.

different than the expected Mendelian distribution of 75% B6, 25% MOLF alleles for an unlinked marker) were subsequently subject to linkage analysis using the panel of 174 individual backcross samples. In addition, flanking markers were typed to confirm positive (linkage) or negative (no linkage) results using the complete panel of individuals. For positive results, chromosomal linkage maps with multiple markers were constructed to refine the localization of the QTL, as described by Welch et al. 1996. Linkage analysis was performed using MAP MANAGER QTB28PPC as described for backcrosses (Manly and Olson 1999; Paterson et al. 1991). Due to the strong effect of sex on atherosclerosis and lipoprotein phenotypes, all analyses were performed separately for males and females. Similar results were obtained using raw or square root-transformed lesion area data. A logarithm of odds (LOD) score of 3.3 was used as the threshold for "significant" linkage (Lander and Kruglyak 1995).

*Statistical analysis:* ANOVA was performed using STATVIEW 5.0 (Abacus Concepts, Inc., Berkeley, CA) for Macintosh computers.

*Sequencing of LOX-1:* Peritoneal macrophages were isolated from C57BL/6J and MOLF/Ei mice. RNA was extracted from the macrophages and reverse-transcribed. The cDNA sequences of *Olr1* (more commonly referred to as LOX-1 in the literature) were determined by polymerase chain reaction using LOX-1-specific primers followed by TA cloning (Shuman 1994) and automated sequencing. The sequences of primers used to amplify the coding region of *Lox-1* were as follows:

Forward, 5'-ATG ACT TTT GAT GAC AAG ATG AAG CCT GCG-3'  
(SEQ ID NO: 9)

Reverse, 5'-CTT CTC ATG GTC TTC TCC AGA ATC TTT AGA-3'  
(SEQ ID NO: 10).

### Results

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The distribution of aortic fatty streak lesion areas among 174 [(MOLF x B6.Ldlr0) X B6.Ldlr0] backcross mice homozygous for the *Ldlr* knockout allele (Mbc-Ldlr0), and the range of values in a set of B6-Ldlr0 controls, is shown in Figure 1. Female Mbc-Ldlr0 mice exhibited 28% larger mean lesion areas than males (mean  $\pm$  SD:  $5.1 \pm 2.2 \times 10^5$  vs.  $3.7 \pm 1.9 \times 10^5 \mu\text{m}^2/\text{section}$ , respectively,  $P < 0.0001$ ). However, there was a broad distribution of lesion values among both female and male Mbc-Ldlr0 mice. The range of lesion areas observed for the B6-Ldlr0 controls was centered around the middle of the distribution curves for both female and male Mbc-Ldlr0 mice, suggesting the presence of both resistance and susceptibility alleles within the B6 genome.

To rule out an effect of *Apoa2*, previously reported to have major effects on HDL cholesterol levels and aortic lesion susceptibility in other genetic crosses (Machleder et al. 1997; Mehrabian et al. 1993), the closely-linked microsatellite marker *D1Mit206* was typed in the panel of 174 Mbc-Ldlr0 mice. No linkage was detected for HDL cholesterol or atherosclerosis susceptibility. The lack of association between lesion areas and genotype at the *Apoa2*-linked marker suggested the presence of novel susceptibility loci segregating among the Mbc-Ldlr0 mice.

To detect candidate linkages for lesion susceptibility, a genome scan was performed using a DNA pooling strategy. The mean lesion areas in Mbc-Ldlr0 mice selected for the "low" pools were  $2.3 \times 10^5$  and  $1.4 \times 10^5 \mu\text{m}^2/\text{section}$  for

females and males, respectively. The mean lesion areas for the "high" pools were  $7.0 \times 10^5$  and  $6.5 \times 10^5$   $\mu\text{m}^2$ /section for females and males, respectively. A total of 88 polymorphic markers were typed, resulting in an average marker spacing of approximately 18 centiMorgans (cM). DNA pooling can usually detect linkage within 30 cM of an allele that is preferentially represented in affected individuals (Collin et al. 1996; Taylor et al. 1994).

Two candidate loci were confirmed by linkage analysis using the complete panel of 174 backcross mice (Table 1). The loci have been designated *Athsql* (Chr 4) and *Athsq2* (Chr 6), for atherosclerosis susceptibility QTL 1 and 2. *Athsql* was supported by a peak LOD score of 6.2 near *D4Mit127* (approximately 77 cM distal to the centromere, as listed in the Mouse Genome Database, MGD) (Fig. 2). Linkage was detected in females only, explaining 32% of the total variance of atherosclerotic lesion areas among females. *Athsq2* was supported by a peak LOD score of 6.7 near *D6Mit110* (62 cM distal to the centromere, as listed in MGD) (Fig. 2). The Chr 6 locus exhibited similar linkage in females (LOD = 3.5, explaining 16% of the variance) and males (LOD = 3.2, explaining 14% of the variance). Female and male LOD plots were coincident, indicating that a single QTL underlies the linkage in both sexes. Confidence intervals defined by a one-unit decrease in the peak LOD score were estimated to be approximately 10 cM for both *Athsql* and *Athsq2*.

The QTL effects on lesion areas and common risk factors for human atherosclerosis are shown in Tables 2 and 3. In females, inheritance of two copies of the B6-derived allele (BB) of *Athsql* resulted in 40% smaller mean lesion area relative to inheritance of one copy of the B6- and

one copy of the MOLF-derived alleles (MB); no effect of genotype was observed in males (Table 2). Conversely, inheritance of the BB genotype at *Athsq2* resulted in 28% (females) and 33% (males) larger mean lesion area relative to inheritance of the MB genotype (Table 3). Plasma total cholesterol, HDL cholesterol and non-HDL-cholesterol levels following feeding of a Western-type diet for two weeks were tested for linkage to the atherosclerosis QTLs; no significant linkages were detected for any of the phenotypes. A small difference in mean HDL cholesterol levels was observed by ANOVA in mice grouped by genotype at *Athsq1* (Table 2). However, the difference was not statistically significant after correcting for multiple testing. In addition, the atherosclerosis-resistant genotype was associated with lower HDL cholesterol levels. This is opposite to what would be expected if the mechanism for atherosclerosis susceptibility determination was through regulation of HDL cholesterol levels. No other effects of the QTLs on plasma cholesterol levels were observed. Similarly, no significant linkages were detected for triglycerides, body weight or basal metabolic index (calculated as body weight divided by the squared nose to anus length) at the atherosclerosis susceptibility QTLs.

Epidemiological studies have shown an association between hyperinsulinemia and coronary atherosclerosis (Bavenholm et al. 1995; Gaudet et al. 1998), as well as clustering of cardiovascular disease risk factors (Bonora et al. 1997; Meigs et al. 2000; Mykkanen et al. 1997). To test for an association between insulin levels and atherosclerosis susceptibility in our mouse model, we compared mean fasting insulin levels in a subset of Mbc-Ldlr0 mice grouped by genotype at the Chr 4 and Chr 6 QTLs. The mice had been fed the Western-type diet for

three months. No significant associations were observed (Tables 2 and 3).

The combined effect of *Athsq1* and *Athsq2* was estimated by comparing mean lesion areas in mice grouped by genotype at both loci (Table 4). Mice carrying both susceptible genotypes, MB at *Athsq1* and BB at *Athsq2*, exhibited two-fold greater lesion area than mice carrying both resistant genotypes (mean  $\pm$  SD:  $6.6 \pm 2.0 \times 10^5$  vs.  $3.2 \pm 1.8 \times 10^5 \mu\text{m}^2/\text{section}$ , respectively). Mice carrying one susceptible and one resistant genotype exhibited intermediate lesion areas. There was no evidence of interaction between the two loci by 2-way ANOVA. These data are consistent with an additive effect of *Athsq1* and *Athsq2* on lesion susceptibility.

Multiple isoforms (sequence variants) of LOX-1, a gene mapped to the region overlapping *Athsq2*, were identified from both C57BL/6J and MOLF/Ei macrophages. Isoforms are different forms of a single gene (can relate to RNA transcripts or protein products). cDNA structures were determined by comparison with published rat (Nagase et al., 1998) and human (Sawamura et al., 1997) sequences. The major isoform found in both mouse strains, Isoform 1, exhibited similar gene structure to rat and human. The conserved structure includes a 5' signal peptide domain, transmembrane domain, leucine zipper motif, unique repetitive region, and a large lectin-like domain. Alignment of the mouse isoform sequences was performed using DIALIGN 2 (Burkhard Morgenstern, 1999). The alignment revealed that novel forms of LOX-1 lacking the transmembrane domain are expressed in MOLF/Ei macrophages but not C57BL/6J.

Sequence alignment of mouse LOX-1 coding regions are

shown in Figure 3A-3C for the following isoforms: B6-Isoform 1 (B24), rat lox-like (SEQ ID NO: 11); MOLF-Isoform 1 (M2), rat lox-like (SEQ ID NO: 12); soluble Isoform 7 (M15) (SEQ ID NO: 13); soluble Isoform 8 (M18) (SEQ ID NO: 15); and soluble Isoform 9 (M17) (SEQ ID NO: 17). The sequences represent the complete coding region of each isoform. B-Isoform 1 is the major isoform derived from strain C57BL/6J. M-Isoform 1 is the major isoform derived from strain MOLF/Ei. Isoforms 7, 8, and 9 were derived from strain MOLF/Ei but not from strain C57BL/6J. B-Isoform 1 and M-isoform 1 contain a transmembrane domain; Isoforms 7, 8, and 9 are soluble and do not contain a transmembrane domain. B-Isoform 1 and M-isoform 1 are 100% identical. Isoforms 7, 8, and 9 are nearly identical to the major form except for the deletions.

The nucleotide and amino acid sequences for nine LOX-1 isoforms are shown in Figures 4-12. The amino acid sequence for isoforms 2, 5, and 6 is the same even though they have different nucleotide sequences. Isoforms 2, 5, and 6 contain only intracellular and membrane-spanning regions but lack any extracellular domains. This occurs because the missing segment, which encodes the lucine zipper in isoform 1, causes a frame shift thereby introducing a stop codon. Isoforms 3 and 4 are membrane-bound.

The alignment of the amino acid sequences of the LOX-1 repeat motifs is shown in Figure 13. Isoforms 2, 5, and 6 are truncated proteins which do not contain repeats. Isoform 9 contains a large deletion which excludes the repeats. The repeat motifs encoded by macrophage-derived isoforms of mouse LOX-1 are aligned with a homologous region encoded by endothelial-derived human LOX-1 in



Figure 13E. A signature motif for the LOX-1 receptor (SEQ ID NO: 39) is identified from this alignment.

5 The repeat units of LOX-1 are predicted to form highly conserved coiled coil structures. The probability plot for Isoform 1 is shown in Figure 14. Since repeats 1, 2, and 3 are in the extracellular domain, they are likely to be involved in intra- or inter-molecular protein  
10 binding. There is precedence for the functional importance of coiled coil structures in the extracellular domains of membrane receptors. Specifically, disruption of the coiled coil structure in the extracellular domain of macrophage scavenger receptors, which also bind and  
15 internalize modified LDL through receptor-mediated endocytosis, results in impaired endocytosis of the ligand (Doi et al. 1994).

### Discussion

20 The *Ldlr* knockout model of atherosclerosis was used to map susceptibility loci to mouse Chrs 4 (*Athsq1*) and 6 (*Athsq2*). *Athsq1* exhibited strong sex-specificity, contributing to disease susceptibility in females but not  
25 males. Together, genotypes at *Athsq1* and *Athsq2* accounted for approximately 50% of the total variance of lesion area among females. The DNA pooling strategy employed in this study allows the detection of independent susceptibility loci that are common among individuals  
30 contributing to a pool. Thus, pooling by phenotype roughly corresponds to pooling by genotype. The inability to detect QTLs contributing to the remaining 50% of the genetic variation of lesion area in this cross is likely due to genetic heterogeneity, small gene effects, and  
35 gene-gene interactions. These results are consistent with

complex inheritance of atherosclerosis susceptibility in the mouse model.

In previous studies, feeding an atherogenic diet to  
5 inbred strains of mice often resulted in marked decreases  
of HDL cholesterol levels in atherosclerosis susceptible  
strains but not resistant strains (Machleder et al. 1997;  
Mehrabian et al. 1993; Paigen et al. 1987, 1989). This  
common finding led to the suggestion that genetic  
10 determinants of HDL cholesterol levels were responsible  
for the differences in atherosclerosis susceptibility.  
However, more recent studies of differential gene  
expression in macrophages and endothelial cells derived  
from resistant and susceptible strains point out that  
15 there are differences in a variety of pathways that could  
influence atherogenesis (Friedman et al. 2000; Shi et al.  
2000).

In the current study, no significant associations were  
20 observed between *Athsq1* or *Athsq2* and plasma lipoprotein  
levels. These results suggest that in a  
hypercholesterolemic model of atherosclerosis, such as  
the *Ldlr* knockout model, variation in disease  
susceptibility is determined by factors independent of  
25 plasma lipoprotein levels. Similarly, genetic studies of  
atherosclerosis in the apolipoprotein E knockout model  
suggest a role for non-lipoprotein-related factors in  
determining the relative susceptibility of different  
mouse strains (Dansky et al. 1999; Grimsditch et al.  
30 2000; Shi et al. 2000). The inability of cholesterol-  
lowering protocols to decrease risk of disease-related  
events in many susceptible humans has highlighted the  
need to develop novel therapeutic approaches. As such,  
the identification of non-lipoprotein-related factors -  
35 such as those involved in inflammation, LDL oxidation,

and macrophage or endothelial cell function - is an area of intense investigation in the atherosclerosis field (Glass and Witztum 2001). Identification of the genes underlying *Athsq1* and *Athsq2* may shed light on novel pathways involved in atherogenesis.

Oxidized LDL is believed to be an essential component of atherogenesis that induces endothelial dysfunction and accumulation of foam cells (Ross 1993). OLR1 protein (also referred to as LOX-1) is a cell-surface receptor expressed in endothelial cells (Sawamura et al. 1997) and macrophages (Nagase et al. 1998) among other cell types; the receptor specifically binds, internalizes, and degrades oxidized LDL but not native LDL (Sawamura et al. 1997). OLR1 was shown to be expressed in atheromatous intima (Kataoka et al. 1999; Yoshida et al. 1998). Comparative sequence analysis of LOX-1, which maps to the region exhibiting peak linkage for *Athsq2* (Depatie et al. 2000; Renedo et al. 2000), revealed multiple isoforms of the LOX-1 receptor in macrophages derived from the C57BL/6J and  $\Delta$ MOLF/Ei strains.

Membrane receptors lacking a transmembrane domain are soluble within the cell and may be targeted for secretion. The secretion of soluble receptors into the circulation provides a mechanism by which cells regulate signal transduction events. Thus, circulating soluble forms of a receptor bind the receptor ligand, prevent binding of the ligand to the membrane-bound receptor and inhibit downstream intracellular signalling events. The binding of oxidized low density lipoproteins to membrane-bound LOX-1 initiates a signal transduction pathway involved in the early stages of atherogenesis. Increasing the level of soluble LOX-1 receptor will increase the binding of LOX-1 ligand to the soluble

receptor, thereby decreasing the binding of ligand to the LOX-1 membrane receptor, thus inhibiting LOX-1 signal transduction. This strategy may be used to prevent and treat atherogenesis.

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The murine localizations of *Athsq1* and *Athsq2* can be used to predict the locations of human candidate susceptibility loci. Distal Chr 4 (*Athsq1*) and distal Chr 6 (*Athsq2*) exhibit extensive homologies with human Chr 1p36-32 and 12p13-12, respectively (Mouse Genome Database, The Jackson Laboratory, Bar Harbor, Maine). The regions of homology flank the confidence interval for each QTL, contain mapping data for more than 50 orthologs per region, and do not overlap any other regions of homology. Thus, Chr 1p36-32 and Chr 12p13-12 are good candidates for focused linkage analyses with densely-spaced markers. Single nucleotide polymorphisms (SNPs) covering the candidate regions have been identified (Cargill et al. 1999; Wang et al. 1998). These markers can be used in disease-association studies (Rubin and Tall 2000) to test the relevance of *Athsq1* and *Athsq2* in human atherosclerosis.

This application discloses novel isolated nucleic acids and their protein products which can be used in the treatment of atherosclerosis and prevention of heart attack and stroke.

**Table 1.** Linkage of lesion susceptibility QTLs to Chr 4 and Chr 6 in Mbc-Ldlr0 mice.

5	Chr <sup>1</sup>	cM	LOD (%VAR) <sup>2</sup>		LOD Combined (N = 174)	QTL symbol
			Males (N = 92)	Females (N = 72-82)		
	4	77	---	6.2 (32%)	---	Athsq1
	6	62	3.2 (14%)	3.5 (18%)	6.7	Athsq2

<sup>1</sup>cM, distance from the centromere in centiMorgans.

10 <sup>2</sup>LOD, logarithm of the odds ratio for linkage; %VAR, an estimate of the percent of the total variance of lesion area explained by the locus.

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**Table 2.** Fatty streak lesion areas, plasma cholesterol levels, and fasting plasma insulin levels in Mbc-Ldlr0 mice grouped by genotype at *D4Mit127*. Values are mean  $\pm$  SD.

Genotype <sup>1</sup>	Lesion area ( $\mu\text{m}^2/\text{section}$ )	Total-C (mg/dl)	HDL-C (mg/dl)	Non-HDL-C (mg/dl)	Insulin (ng/ml)
Females					
BB (N = 32)	$3.6 \pm 1.8 \times 10^5$	$344 \pm 43$	$51 \pm 13$ $59 \pm$	$295 \pm 47$ $284 \pm 52$	$1.32 \pm 1.0$ (n=15)
MB (N = 40)	$6.1 \pm 2.0 \times 10^5$	$341 \pm 55$	$16$		$1.06 \pm 0.67$ (n=7)
Males					
BB (N = 39)	$3.6 \pm 2.2 \times 10^5$	$384 \pm 57$	$71 \pm 17$ $75 \pm 15$	$311 \pm 59$ $291 \pm 54$	$3.16 \pm 1.66$ (n=27)
MB (N = 48)	$3.6 \pm 2.0 \times 10^5$	$366 \pm 56$			$3.66 \pm 2.61$ (n=10)

<sup>1</sup>BB, homozygous for C57BL/6J alleles; MB, heterozygous for C57BL/6J and MOLF alleles.

<sup>\*</sup>P < 0.0001 vs. BB.

<sup>\*\*</sup>P < 0.03 vs. BB.

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**Table 3.** Fatty streak lesion areas, plasma cholesterol levels, and fasting plasma insulin levels in Mbc-Ldlr0 mice grouped by genotype at *D6Mit110*. Values are mean  $\pm$  SD.

Genotype <sup>1</sup>	Lesion area ( $\mu\text{m}^2/\text{section}$ )	Total-C (mg/dl)	HDL-C (mg/dl)	Non-HDL-C (mg/dl)	Insulin (ng/ml)
Females					
BB (N = 43)	5.8 $\pm$ 2.0 x10 <sup>5</sup>	342 $\pm$ 45	48 $\pm$ 16	292 $\pm$ 42 286 $\pm$ 54	1.12 $\pm$ 0.79 (n=16)
MB (N = 39)	4.2 $\pm$ 2.1 x10 <sup>5*</sup>	341 $\pm$ 51	53 $\pm$ 14		1.37 $\pm$ 1.12 (n=7)
Males					
BB (N = 47)	4.4 $\pm$ 1.8 x10 <sup>5</sup>	379 $\pm$ 50	66 $\pm$ 17	312 $\pm$ 47 287 $\pm$ 61	3.42 $\pm$ 2.21 (n=17)
MB (N = 45)	2.9 $\pm$ 1.8 x10 <sup>5**</sup>	364 $\pm$ 60	72 $\pm$ 12		3.18 $\pm$ 1.77 (n=20)

<sup>1</sup>BB, homozygous for C57BL/6J alleles; MB, heterozygous for C57BL/6J and MOLF alleles.

\*P < 0.0009 vs. BB.

\*\*P < 0.0002 vs. BB.

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**Table 4.** Combined effects of *Athsq1* and *Athsq2* on lesion areas in 72 female Mbc-Ldlr0 mice. Values are mean  $\pm$  SD in  $\mu\text{m}^2$ /section.

	QTL, genotyp e <sup>1</sup>	<i>Athsq2</i> , BB	<i>Athsq2</i> , BM
5			
10	<i>Athsq1</i> , MB	$6.6 \pm 2.0 \times 10^5$ (N = 22)	$5.3 \pm 2.0 \times 10^5$ (N = 16)
15	<i>Athsq1</i> , BB	$4.1 \pm 1.4 \times 10^5$ (N = 11)	$3.2 \pm 1.8 \times 10^5$ (N = 19)
	<sup>1</sup> BB, homozygous for C57BL/6J alleles; MB, heterozygous for C57BL/6J and MOLF alleles.		

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